Virology **262,** 398–407 (1999)
Article ID viro.1999.9912, available online at http://www.idealibrary.com on IDEAL®

Diarrhea Induction by Rotavirus NSP4 in the Homologous Mouse Model System

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Received March 31, 1999; returned to author for revision April 27, 1999; accepted July 20, 1999

Comparison of the NSP4 amino acid sequences from 31 strains of mammalian rotaviruses revealed the presence of four distinct NSP4 alleles; i.e., the Wa, KUN, AU-1, and EW alleles. The EW allele consists only of NSP4s from murine rotavirus strains and is divergent from other NSP4 alleles from the evolutionary perspective. There have been conflicting reports regarding the enterotoxigenic activity of NSP4 in the mouse model system; heterologous simian and porcine rotavirus NSP4s function as an enterotoxin in mice, while a homologous EC NSP4 does not play a dominant role as an enterotoxin in the cystic fibrosis conductance regulator knockout mice. To further examine the enterotoxigenic activity of NSP4, we expressed in *Escherichia coli* a recombinant protein consisting of glutathione S-transferase and amino acid residues 86–175 of the EW NSP4. We found that this fusion protein caused diarrhea in the majority (8/14) of 5- to 6-day-old CD1 mice. This study confirmed and extended that group A rotavirus NSP4s were able to induce diarrhea in neonatal mice and had an enterotoxigenic activity.

INTRODUCTION

Rotaviruses are recognized as the most important cause of severe diarrhea in infants and young animals of many mammalian species (Kapikian and Chanock, 1996). The rotavirus genome, which is contained in a triple-layered capsid, consists of 11 segments of double-stranded RNA, with six genome segments coding for the structural proteins (VP1, VP2, VP3, VP4, VP6, and VP7) and five segments coding for the nonstructural proteins (NSP1-5) (Estes, 1996). Ball *et al.* (1996) reported recently that the baculovirus-expressed NSP4 from the simian rotavirus strain SA11 could function as a viral enterotoxin in the mouse model system. Furthermore, Zhang *et al.* (1998) reported that the baculovirus-expressed NSP4 from virulent porcine rotavirus strain OSU-v was capable of inducing diarrhea in neonatal mice, whereas the baculovirus-expressed NSP4 from attenuated porcine rotavirus strain OSU-a was not. Ball *et al.* (1996) and Zhang *et al.* (1998) gave a plausible explanation for the mechanism of diarrhea induction in mice; the NSP4 could induce diarrhea by stimulating chloride secretion through a calcium-dependent signaling pathway after binding to the putative receptor on the intestinal epithelium.

On the other hand, Angel *et al.* (1998) observed that the murine rotavirus strain EC particles could induce diarrhea in CFTR (cystic fibrosis conductance regulator) knockout neonatal mice that lacked the calcium-modulated chloride channel in their intestinal epithelium. They concluded that NSP4 did not play a dominant role in inducing diarrhea. Thus, the enterotoxigenic activity of NSP4 remains to be confirmed.

By comparing group A rotavirus NSP4 amino acid sequences from 13 human, two porcine, two bovine, two simian, two feline, and one canine strains, we have previously identified three distinct NSP4 alleles, i.e., the Wa, KUN, and AU-1 alleles, which are represented by human rotavirus strains Wa, KUN, and AU-1, respectively (Horie *et al.,* 1997). The SA11 and OSU NSP4s were classified into the KUN and Wa alleles, respectively. These heterologous NSP4s were able to induce diarrhea in neonatal mice. It was interesting to know whether the homologous NSP4 from murine rotavirus was classified into any one of the three alleles. Moreover, NSP4s from the equine and lapine rotaviruses have not yet been sequenced in NSP4s from the group A rotavirus strains isolated from the mammalian species. It was also not clear whether NSP4s from the equine and lapine rotaviruses were classified into any one of the three alleles. Thus, we sequenced group A rotavirus NSP4s from two equine, one lapine, and one murine rotavirus strain.

In this study, we determined these four NSP4 sequences, and analysed them together with 27 group A rotavirus NSP4 sequences available in the DNA databases. We then expressed the murine rotavirus EW NSP4 in *Escherichia coli* and gave it to 5- to 6-day-old CD1 mice to examine whether the mice got diarrhea.

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0042-6822/99 $30.00

TABLE 1

Rotavirus Strains Used in This Study

Strain	Host species	G type	P type	Subgroup	Accession number	Reference
Wa	Human	1	1A[8]	II	K02032	Okada *et al.,* 1984
ST3	Human	4	2A[6]	II	U59110	Kirkwood and Palombo, 1997
RV3	Human	3	2A[6]	II	U42628	Kirkwood *et al.,* 1996
RV4	Human	1	1A[8]	II	U59108	Kirkwood and Palombo, 1997
AU32	Human	9	1A[8]	II	D88830	Horie *et al.,* 1997
M37	Human	1	2A[6]	II	U59109	Kirkwood and Palombo, 1997
116E	Human	9	5[11]	II	U78558	—[a]
VA70	Human	4	1A[8]	II	U83798	Kirkwood and Palombo, 1997
OSU	Porcine	5	9[7]	I	D88831	Horie *et al.,* 1997
YM	Porcine	11	9[7]	I	X69485	Lopez and Arias, 1993
KUN	Human	2	1B[4]	I	D88829	Horie *et al.,* 1997
RV5	Human	2	1B[4]	I	U59103	Kirkwood and Palombo, 1997
S2	Human	2	1B[4]	I	U59104	Kirkwood and Palombo, 1997
1076	Human	2	2A[6]	I	U59105	Kirkwood and Palombo, 1997
E210	Human	2	1B[4]	II	U59107	Kirkwood and Palombo, 1997
A28	Human	10	ND[b]	I	D01145	Ballard *et al.,* 1992
US1205	Human	9	2A[6]	ND	AF079358	—
UK	Bovine	6	7[5]	I	M21885	Baybutt *et al.,* 1984
NCDV	Bovine	6	6[1]	I	X06806	Powell *et al.,* 1988
B-I	Equine	5	[7]	ND	AB005473	This study
HI23	Equine	ND	ND	ND	AB005474	This study
SA11	Simian	3	[2]	I	K01138	Both *et al.,* 1983
AU-1	Human	3	3[9]	I	D89873	Horie *et al.,* 1997
FRV-1	Feline	3	3[9]	I	D89874	Horie *et al.,* 1997
RS15	Canine	3	5[3]	I	D88832	Horie *et al.,* 1997
FRV64	Feline	3	5[3]	I	D88833	Horie *et al.,* 1997
R2	Lapine	3	[14]	II	AB005472	This study
RRV	Simian	3	5[3]	I	L41247	—
EW	Murine	3	[16]	nonI/II	AB003805	This study
EHP	Murine	3	[20]	ND	U96336	Angel *et al.,* 1998
EC	Murine	3	[16]	ND	U96337	Angel *et al.,* 1998

[a] Indicates unpublished data.
[b] Not described.

RESULTS

Phylogenetic relationships of the group A rotavirus NSP4 amino acid sequences

We sequenced the NSP4s from one murine (EW), two equine (B-I and HI23), and one lapine (R2) rotavirus strain and analyzed them together with 27 NSP4 sequences available in the DNA databases (Table 1); the NSP4 amino acid sequences analyzed in this study were derived from 16 human, 2 porcine, 2 bovine, 2 equine, 2 simian, 2 feline, 1 canine, 1 lapine, and 3 murine rotavirus strains. The overall amino acid identities were greater than 60% (Table 2). To more accurately infer phylogenetic relationships, the evolutionary distances between strains were computed for each pair of the NSP4 amino acid sequences using the Dayhoff PAM 001 matrix (Dayhoff *et al.,* 1978). The evolutionary distances ranged from 0.5% (between AU-1 and FRV-1, between KUN and S2, and between KUN and E210) to 53.2% (between EW and M37) (Table 2). A closer look at the evolutionary distances allowed identification of the fourth NSP4 allele, which was represented by the murine rotavirus strain EW, in addition to the Wa, KUN, and AU-1 NSP4 alleles. The evolutionary distances within alleles were less than 10%. The EW allele included the NSP4s from two other murine rotavirus strains, EC and EHP. Of the nine NSP4s added in this study, two human rotavirus strains, 116E and VA70, NSP4s were classified into the Wa allele. Another human rotavirus strain US1205 NSP4 was classified into the KUN allele. Two equine rotavirus strains, B-I and HI23, NSP4s, and one lapine rotavirus strain, R2 NSP4, were classified into the AU-1 allele. Among the Wa allele, represented by the human strain Wa, were seven other human strains and two porcine strains. Among the KUN allele, represented by the human strain KUN, were six other human strains, two bovine strains, two equine strains, and the simian SA11 strain. The third allele, designated as the AU-1 allele, included the human AU-1 strain, feline FRV-1 and FRV64, canine RS15, simian RRV, and lapine R2. In respect to host species, while the EW allele is homogeneous, the Wa, KUN, and AU-1 alleles are heterogeneous.

TABLE 2

Amino Acid Identities (above Diagonal) and Evolutionary Distances (below Diagonal) between NSP4s of Various Human and Animal Rotavirus Strains

	Wa	AU32	RV3	RV4	ST3	M37	OSU	YM	VA70	116E	KUN	US1205	RV5	S2	1076	E210	A28	UK	NCDV	SA11	B-I	HI23	AU1	FRV1	FRV64	RS15	RRV	R2	EW	EC	EHP
Wa	—	95	96	96	95	97	94	95	97	95	84	84	82	83	82	84	83	84	85	82	83	84	84	85	82	83	84	83	61	61	61
AU32	4.4	—	94	94	92	93	93	93	94	93	83	83	82	82	82	83	82	84	85	81	83	84	85	86	82	84	85	85	61	61	61
RV3	3.3	6.1	—	95	94	95	92	94	94	95	84	83	82	83	81	84	83	82	83	82	83	83	82	83	81	81	82	82	62	62	62
RV4	3.3	5.5	4.9	—	95	95	93	95	97	94	84	84	82	83	82	85	84	84	85	83	83	85	84	85	82	83	84	83	62	61	61
ST3	4.4	7.9	6.1	4.4	—	94	91	93	96	93	82	82	81	82	81	82	81	82	82	80	81	82	82	83	80	81	82	82	61	61	61
M37	2.7	6.7	4.9	4.4	5.5	—	92	93	96	93	84	84	82	83	82	85	83	85	85	82	82	83	83	83	81	82	83	82	61	61	60
OSU	5.5	7.3	7.2	6.6	8.4	7.2	—	95	93	95	85	85	84	84	83	85	83	85	86	83	83	83	82	83	82	81	82	82	61	61	61
YM	4.9	7.2	6.6	4.9	7.8	7.2	5.4	—	95	95	83	83	82	82	82	83	83	83	84	82	83	85	82	83	80	82	82	82	62	62	61
VA70	2.7	6.2	5.6	2.8	3.7	3.9	7.3	5.1	—	94	83	83	82	83	82	84	83	83	84	82	83	85	83	84	81	83	83	83	62	62	61
116E	4.4	6.7	4.9	5.5	7.3	6.7	5	5.5	6.2	—	84	84	82	83	82	85	85	83	83	82	83	83	83	83	82	82	83	83	62	62	61
KUN	16.4	17.6	16.4	16.3	18.4	16.4	15	18.2	17.3	16.4	—	99	95	99	94	99	94	94	94	94	93	93	83	84	84	81	83	84	63	63	63
US1205	16.3	17.5	16.9	16.2	19	16.4	15	18.2	17.2	16.4	1.1	—	94	98	93	99	94	97	94	93	93	93	83	84	84	81	83	84	61	63	62
RV5	18.2	18.8	18.2	18.1	20.2	18.3	16.1	19.5	19.1	18.2	4.5	5.6	—	95	93	95	93	94	94	94	93	93	83	83	83	80	82	83	63	63	63
S2	17	18.2	17	16.9	19.1	17.1	15.7	18.9	17.9	17	0.5	1.6	5	—	93	99	94	93	94	94	92	92	83	83	83	80	82	83	63	63	63
1076	18.3	18.9	19.6	18.2	20	18.4	16.8	18.9	19.2	18.3	6.2	7.3	7.2	6.7	—	93	91	92	91	91	91	91	82	83	81	79	82	82	62	63	61
E210	15.7	16.7	16.3	15.6	18.3	15.8	14.4	17.5	16.6	15.7	0.5	0.5	5	0.8	6.7	—	95	94	95	94	93	93	84	85	85	81	83	85	63	63	63
A28	17	18.2	17.6	16.3	19.7	17.1	16.8	17.7	17.9	15.7	5.6	5.6	6.8	6.2	8.6	5	—	93	94	92	93	93	84	85	84	81	83	85	62	63	62
UK	16.3	17.4	16.3	16.2	18.3	16.3	14.3	17.5	17.2	16.3	2.2	3.3	2.2	2.7	6.2	2.7	4.5	—	98	92	94	95	86	86	84	83	85	85	63	62	63
NCDV	15.8	15.7	17.6	15.7	18.4	15.8	14.5	17	16.6	17.1	5.6	5.6	5.7	6.2	8.5	5	5.6	2.6	—	92	94	95	86	86	84	83	85	85	62	62	62
SA11	18.8	19.4	18.8	17.4	20.9	18.2	17.2	18.8	18.4	18.9	5.5	6.7	6.2	6.1	9.1	6.1	7.9	3.9	8	—	90	91	82	82	83	81	81	82	62	62	61
B-I	17	16.9	17.5	16.9	19.6	18.4	17.5	18.2	17.8	17	7.2	7.2	7.3	7.8	9	6.6	6.7	6.1	5.5	9.6	—	94	86	86	83	83	84	86	66	64	65
HI23	16.5	16.4	17.7	15.1	19.1	17.2	17	16.4	16.1	17.7	7.3	7.2	7.4	7.9	9.1	6.7	6.7	6.1	4.4	8.5	5.5	—	85	86	83	83	85	85	63	62	62
AU1	17.2	15.8	19	17.1	19.2	18.6	18.5	19	18	17.9	17.6	17.5	18.3	18.3	19.6	16.9	17.2	16.9	15.2	19.5	15.1	15.6	—	99	93	94	95	97	63	64	63
FRV1	16.5	15.2	18.4	16.5	18.6	17.9	17.8	18.4	17.4	17.3	17	16.9	17.6	17.7	18.9	16.3	16.6	16.3	14.5	18.9	14.5	15	0.5	—	93	94	96	97	63	65	63
FRV64	19.9	19.8	20.5	19.8	22	20.6	19.2	21.8	20.9	19.4	17.2	17.1	18.7	17.9	21.2	16.5	17.5	16.5	17.4	18.3	18	18.5	6.7	7.3	—	92	92	93	63	64	63
RS15	18.3	17	20.1	17.6	20.3	19.7	19.6	19.6	18.6	19	20.8	20.7	21.4	21.4	22.8	20	20.4	20	18.3	20.9	18.2	18.1	5.5	6.1	7.9	—	93	93	63	63	62
RRV	17.1	15.8	19	17	19.2	18.5	18.4	19	18	17.8	18.2	18.1	18.8	18.9	20.2	17.5	17.8	17.5	15.7	20.1	17	16.2	4.9	4.4	7.9	7.2	—	96	63	63	62
R2	17.7	16.4	19.6	17.6	19.8	19.1	19	19.6	18.6	18.4	17.5	17.4	18.1	18.2	19.4	16.8	17.1	16.8	15.7	19.4	15	15.5	3.3	2.7	7.3	6.6	3.8	—	64	65	63
EW	50.4	50	48.3	51	52.1	53.2	49.1	48.4	51.2	48.9	49	50.1	48.5	48.9	51	49.3	50.6	48	50.6	50.8	44.9	49	48.6	48.6	50.5	49	49.6	48.7	—	95	97
EC	50.4	50.3	48.3	50.2	50.6	51.7	48.8	49.2	50.5	48.9	46.7	47.8	48.4	46.6	49.1	47	49.2	46.2	50.7	50.1	46.6	50	47.5	47.6	48.2	49	48.5	47.6	6.2	—	95
EHP	51.2	50.5	49.1	51	51.4	52.5	49.8	49.6	51.3	49.7	48.5	49.5	47.9	48.3	50.6	48.3	50.3	47.4	49.8	51	44.5	49.4	47.9	47.9	49.9	49.3	48.9	48	5	5	—

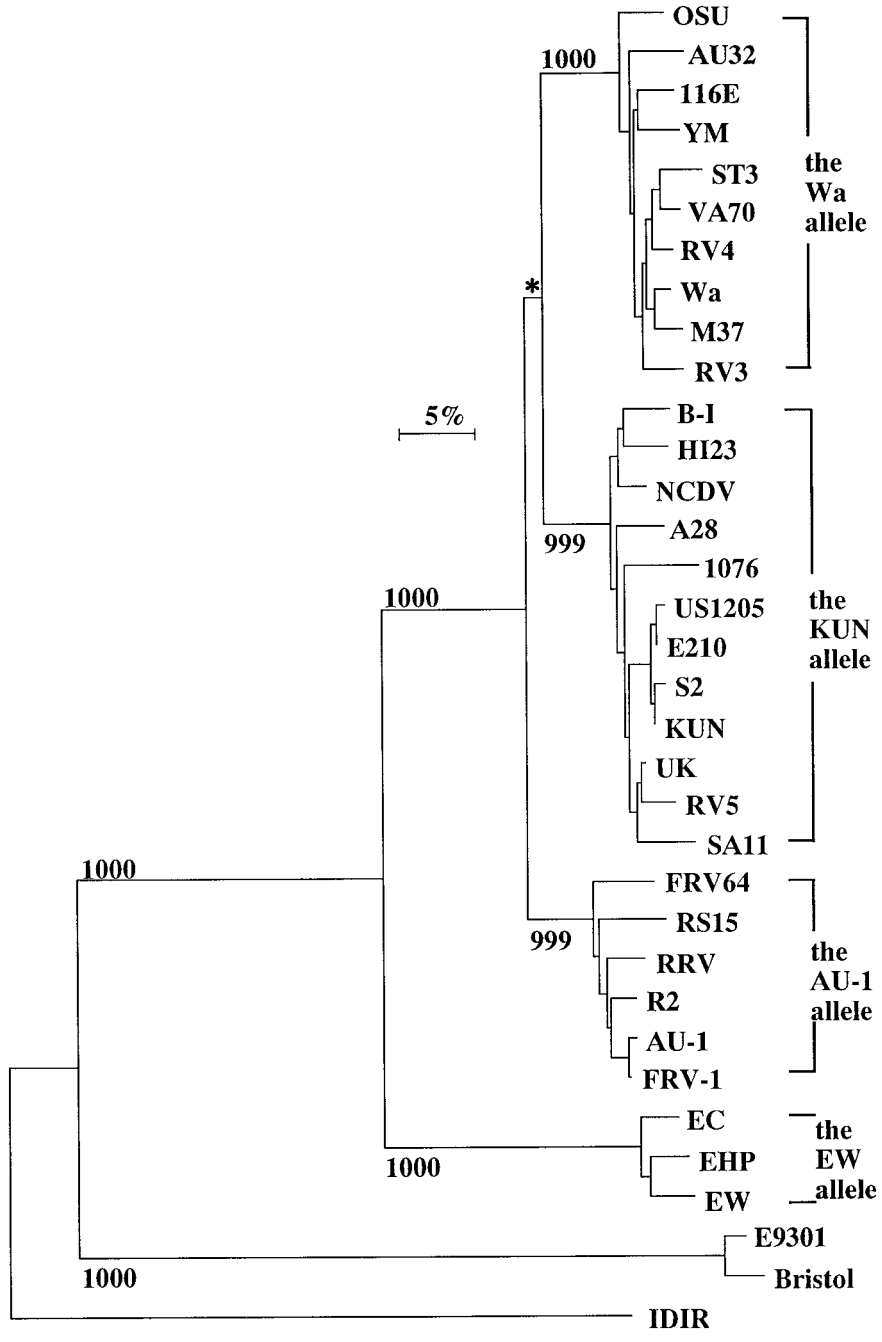

FIG. 1. Phylogenetic tree for NSP4s constructed by the neighbor-joining method of Saitou and Nei (1987). The horizontal distance connecting two NSP4s is proportional to the evolutionary distance between these two NSP4s. The evolutionary distance is expressed as the number of amino acid substitutions per 100 sites (percentage divergence). Bootstrap probabilities equal to or greater than 90% (>900 of 1000 trials) are indicated. The bootstrap probability at the asterisk node (*) is 66.8%. E9301, Bristol, and IDIR are the groups C and B rotavirus strains and were added as outgroups to root the phylogenetic tree.

The evolutionary distances between the Wa and KUN allele, between the Wa and AU-1 allele, and between the KUN and AU-1 allele ranged from 14.3 to 22.8%, while the evolutionary distances between the EW allele and the other three alleles ranged from 44.5 to 53.2%. These suggest that the EW allele is divergent from the other three NSP4 alleles from the evolutionary perspective.

A phylogenetic tree was constructed by the neighbor-joining method using the NSP4s from group C rotaviruses (the Ehime 9301 and Bristol strains) and group B rotavirus (the IDIR strain) as outgroups and confirmed four major lineages corresponding to four distinct NSP4 alleles (Fig. 1). Although divergence into the Wa, KUN, and AU-1 alleles occurred at about the same time during evolution, diver-

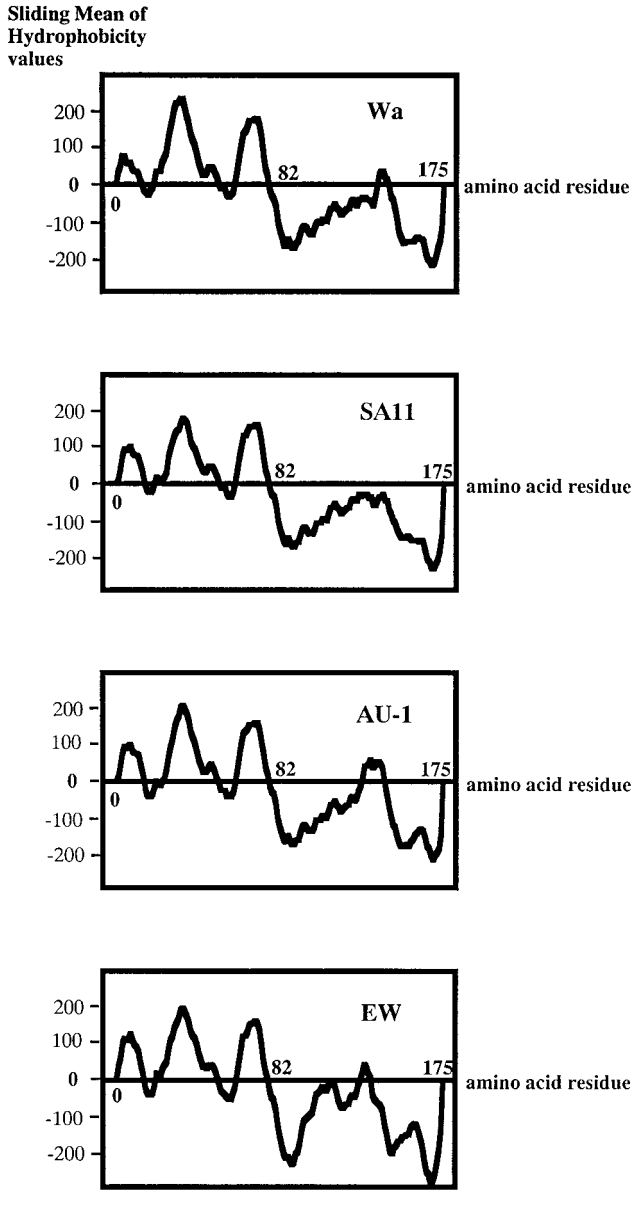

FIG. 2. Hydrophobicity profiles for the Wa NSP4 representing the Wa allele, the SA11 NSP4 representing the KUN allele, the AU-1 NSP4 representing the AU-1 allele, and the EW NSP4 representing the EW allele constructed by the Kyte–Doolittle algorithm.

gence into the EW allele occurred significantly prior to that into the other group A rotavirus alleles (Fig. 1).

Hydrophobicity profiles of the group A rotavirus NSP4 amino acid sequences

The hydrophobicity profiles predicted by the Kyte–Doolittle algorithm for the group A rotavirus NSP4 sequences revealed that the conformation of the NSP4s was consistent irrespective of the alleles or host species from which the NSP4s were derived, thus an amino-terminal half (amino acid residues 1–82) being hydrophobic, a carboxy-terminal half (amino acid residues 83–175) being hydrophilic and in the middle region of the carboxy-terminal half being a relatively hydrophobic domain (Fig. 2).

Expression and confirmation of the GST-EW NSP4$_{86\text{-}175}$ fusion protein and the GST protein

Because we initially failed to express the full-length NSP4 as a GST fusion protein, we expressed the EW NSP4$_{86\text{-}175}$ as a GST fusion protein. The GST-EW NSP4$_{86\text{-}175}$ fusion protein in *E. coli* extracts was readily visible as a 37-kDa band upon SDS–PAGE (Fig. 3). The soluble form of the fusion protein in *E. coli* extracts was expressed at approximately a 100-fold lower level compared to the insoluble form (Fig. 3). However, the fusion protein was expressed in large quantities in *E. coli,* so we put only the soluble form to use and could obtain approximately 5 mg from 1 liter of bacterial cultures. On the other hand, the GST protein was essentially soluble and migrated with an apparent molecular weight of 26 kDa upon SDS–PAGE (data not shown). Approximately 20 mg of the GST protein were obtained from 1 liter of bacterial cultures. Comparison of Coomassie brilliant R-250-stained gel (the upper panel) and Western blot (the lower panel) in Fig. 4 indicated that electrophoresed proteins contained a GST molecule, suggesting that these proteins were the GST fusion protein or the GST protein.

Diarrhea induction in neonatal mice

Intraperitoneal administration of 1 nmol of the GST-EW NSP4$_{86\text{-}175}$ fusion protein induced watery diarrhea in 57.1% (8/14) of the 5- to 6-day-old CD1 mice, whereas 8.3% (1/12) and 10% (1/10) of the 5- to 6-day-old CD1 mice had watery diarrhea after intraperitoneal administration of 1 nmol of the GST protein and 50 μl of the glutathione elution buffer, respectively. Ordinary stool was observed

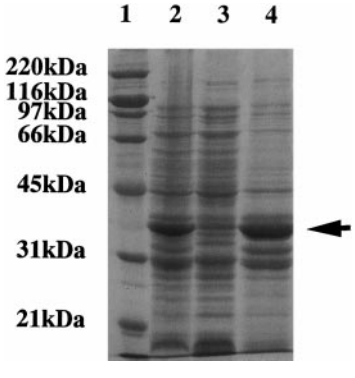

FIG. 3. Subcellular localization of GST-EW NSP4$_{86\text{-}175}$ fusion protein in *E. coli* extracts. Lane 1, marker proteins of known molecular weights. Lane 2, whole cell lysates containing the GST-EW NSP4$_{86\text{-}175}$ fusion protein. Lane 3, the soluble (supernatant) fraction prepared by sonication and centrifugation of the whole cells containing the GST-EW NSP4$_{86\text{-}175}$ fusion protein. Lane 4, the insoluble (pellet) fraction prepared by sonication and centrifugation of the whole cells containing the GST-EW NSP4$_{86\text{-}175}$ fusion protein. The solid arrow indicates the position of the GST-EW NSP4$_{86\text{-}175}$ fusion protein.

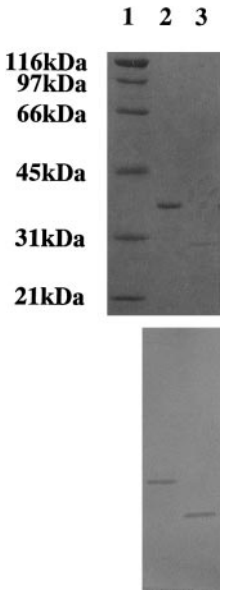

FIG. 4. Confirmation of the GST-EW NSP4$_{86\text{-}175}$ fusion protein and GST protein purified from *E. coli* extracts. Aliquots of 0.05 nmol of the purified GST-EW NSP4$_{86\text{-}175}$ fusion protein and the purified GST protein were separated on a 10% SDS–polyacrylamide gel followed by staining with Coomassie brilliant blue R-250 (the upper panel). Lane 1, markers; Lane 2, 0.05 nmol (corresponding to 0.25 μg) of the purified GST-EW NSP4$_{86\text{-}175}$ fusion protein; Lane 3, 0.05 nmol (corresponding to 0.17 μg) of the purified GST protein. A Western blot corresponding to the upper panel with an anti-GST antibody (the lower panel). Bands were found at the positions where the GST-EW NSP4$_{86\text{-}175}$ fusion protein and the GST protein migrated on SDS–PAGE.

with the GST-EW NSP4$_{86\text{-}175}$ fusion protein in 14.2% (2/14) of the CD1 neonatal mice, whereas 58.3% (7/12) and 70% (7/10) of the CD1 neonatal mice had ordinary stool after intraperitoneal administration of the GST protein and the glutathione elution buffer, respectively (Table 3). The effect of the GST protein on diarrhea induction in CD1 neonatal mice was almost equal to that of the glutathione elution buffer, suggesting that the GST protein had no effect on diarrhea induction in CD1 neonatal mice. Among the diarrheogenic effect of the GST-EW NSP4$_{86\text{-}175}$ fusion protein, the GST protein and the glutathione elution buffer in the 5- to 6-day-old CD1 mice was observed a significant difference; significance, expressed by a Kruskal–Wallis rank test, was 0.0036. These suggest that the GST-EW NSP4$_{86\text{-}175}$ fusion protein can induce diarrhea in CD1 neonatal mice.

Six of eight neonatal mice with watery diarrhea, to which the GST-EW NSP4$_{86\text{-}175}$ fusion protein was administered, had diarrhea within 3 h after administration. Each of two neonatal mice with watery diarrhea, to which the GST protein or the glutathione elution buffer were administered, had diarrhea 6 and 24 h after administration.

DISCUSSION

Phylogenetic analyses revealed that the SA11 and OSU NSP4s belonged to the KUN and Wa alleles, respectively, which were evolutionarily divergent from the EW allele consisting only of the NSP4s from murine rotavirus strains. Thus, the experiment, which is carried out with the EW NSP4 derived from a murine rotavirus strain on neonatal mice, should give a better understanding of the role of the NSP4 in mice. We expressed the EW NSP4$_{86\text{-}175}$ as a GST fusion protein to carry out the experiment because amino acid residues 114–135 of the NSP4 was cytotoxic and increased intracellular calcium in eukaryotic cells (Tian *et al.,* 1995). The GST-EW NSP4$_{86\text{-}175}$ fusion protein could induce diarrhea in CD1 neonatal mice and the GST protein had no effect on diarrhea induction in CD1 neonatal mice. Therefore, it was the EW NSP4$_{86\text{-}175}$ that had an enterotoxigenic activity.

Ball *et al.* (1996) reported that intraperitoneal administration of 0.1 nmol of a full-length SA11 NSP4 induced diarrhea in 60% (6/10) of 6- to 7-day-old CD1 mice. Zhang *et al.* (1998) have also reported that 56.5% (13/23) of 6- to 7-day-old CD1 mice had diarrhea after intraperitoneal administration of 0.25 nmol of a full-length virulent OSU-v NSP4. Similarly, we observed that intraperitoneal administration of 1 nmol of the EW NSP4$_{86\text{-}175}$ induced diarrhea in 57.1% (8/14) of 5- to 6-day-old CD1 mice. Furthermore, Ball *et al.* (1996) reported that the full-length SA11 NSP4 induced diarrhea 1 to 4 h after administration. This kinetics of diarrhea induction agrees with our observation that the EW NSP4$_{86\text{-}175}$ induced diarrhea within 3 h after administration in 6 of 8 CD1 neonatal mice with watery diarrhea. Thus, the effect of the EW NSP4$_{86\text{-}175}$ on CD1 neonatal mice in diarrhea induction is similar to that of the SA11 NSP4.

Ball *et al.* (1996) reported that intraperitoneal administration of 100 nmol of the SA11 NSP4$_{114\text{-}135}$ induced diarrhea in 68.7% (11/16) of CD1 neonatal mice and the disease response to the peptide was dose-dependent ($\chi^2_{\text{trend}} = 9.98$, $P = 0.0016$) with a 50% diarrheal dose of 79 nmol. Thus, we can reasonably extrapolate that 1 nmol of the SA11 NSP4$_{114\text{-}135}$ should minimally induce diarrhea in mice. Furthermore, Ball *et al.* (1996) reported that 1 nmol of the full-length SA11 NSP4 induced diarrhea in 100% (10/10) of mice. We observed that 57.1% (8/14) of CD1 neonatal mice had diarrhea after intraperitoneal administration of 1 nmol of the EW NSP4$_{86\text{-}175}$

TABLE 3

Diarrhea Induction in CD1 Mice

	GST-EW NSP4$_{86\text{-}175}$	GST	Glutathione elution buffer
Watery diarrhea	8	1	1
Loose yellow stool	4	4	2
Ordinary stool	2	7	7

Note. The data are presented as the number of mice that have watery diarrhea, loose yellow stool, or ordinary stool. $P = 0.0036$ (Kruskal–Wallis rank test).

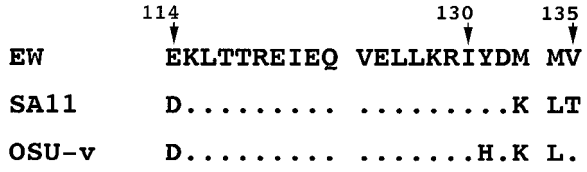

FIG. 5. Predicted amino acid sequence of residues 114–135 of the EW NSP4. The corresponding amino acid sequences of the SA11 (Ball *et al.,* 1996) and OSU-v (Zhang *et al.,* 1998) NSP4s are shown below that of the EW NSP4 only where they differ.

(Table 3). Thus, the enterotoxigenic activity of the NSP4 is roughly proportional to the size of the NSP4 peptide. In general, as a protein becomes small, the protein does not fold into the native conformation (Alberts *et al.,* 1994); hence it is less likely that the $NSP4_{86\text{-}175}$ or $NSP4_{114\text{-}135}$ peptides fold into the native conformation of the full-length NSP4. Therefore, these peptides were presumably less diarrheogenic than the full-length NSP4. Moreover, because the GST-EW $NSP4_{86\text{-}175}$ is a fusion protein, the EW $NSP4_{86\text{-}175}$ peptide might further change the native conformation.

Ball *et al.* (1996) have reported that the SA11 $NSP4_{90\text{-}123}$ peptide, which overlaps the 114–135 peptide by nine residues and is longer than the 114–135 peptide, showed less response than the SA11 $NSP4_{114\text{-}135}$ peptide in diarrhea induction of CD1 neonatal mice. This reduced response suggests that the critical domain of the NSP4 in diarrhea induction is located in amino acid residues 114–135 of the NSP4. Furthermore, it is noteworthy that amino acid sequences of the $NSP4_{114\text{-}130}$ peptide are best conserved, whereas the $NSP4_{131\text{-}135}$ peptide shows the high sequence variation in the amino acid sequence of the NSP4 (Horie *et al.,* 1997). Amino acid sequences at residues 115–130 are completely conserved among the EW, SA11, and OSU-v NSP4s (Fig. 5). Aspartic acid at position 114 of the SA11 and OSU-v NSP4s is replaced with the equivalent amino acid in their physico-chemical nature, glutamic acid, at position 114 of the EW NSP4 (Fig. 5). On the other hand, the amino acid residues at position 131, 133, and 135 are not equivalent in their physicochemical nature among the three strains NSP4s because tyrosine is an uncharged polar amino acid whereas histidine is a positively charged polar amino acid, and methionine and valine are nonpolar whereas lysine and threonine are polar (Fig. 5). The EW, SA11, and OSU NSP4s are highly conserved at residues 114–130 and highly variable at residues 131–135. Therefore, amino acid residues 114–130 of the NSP4 are a candidate that binds to the putative receptor on the intestinal epithelium and induces diarrhea in neonatal mice.

The enterotoxigenic activity of 1 nmol of the NSP4 is roughly proportional to the size of the NSP4 peptide. Furthermore, 100% of CD1 neonatal mice had diarrhea after administration of 1 nmol of the full-length NSP4 (Ball *et al.,* 1996). We conclude that the NSP4 can induce diarrhea alone as a rotavirus enterotoxin in CD1 neonatal mice. This is apparently inconsistent with Angel's report, in which the homologous murine rotavirus strain EC induced diarrhea in CFTR knockout mice (Angel *et al.,* 1998). It may be due to an alternative calcium-modulated intestinal chloride channel in CFTR knockout mice, as Angel *et al.* (1998) suggested. Another explanation is that the homologous murine rotavirus particles induced diarrhea in CFTR knockout mice according to the conventional mechanism. The murine rotaviruses replicate efficiently in the small intestine of CFTR knockout mice and then rotavirus-infected villus enterocytes are destroyed. The loss of large numbers of villus absorptive cells leads to diarrhea. In this case, even if the murine rotavirus NSP4 can not function as an enterotoxin in CFTR knockout mice because of the absence of a calcium-modulated chloride channel in their intestinal epithelium, diarrhea can be observed in neonatal CFTR knockout mice.

We observed that 6 of 8 neonatal mice with watery diarrhea, to which the GST-EW $NSP4_{86\text{-}175}$ fusion protein was administered, had diarrhea within 3 h after administration. This kinetics of diarrhea induction was much earlier than that observed in natural rotavirus infection in neonatal mice (Angel *et al.,* 1998). These can allow us to speculate that the NSP4 plays an important role in inducing diarrhea at the early stage of the natural history of rotavirus illness. After efficient replication of rotaviruses in the small intestine, it is supposed that diarrhea can be induced by both the conventional mechanism and the NSP4.

The phylogenetic analysis for 31 NSP4s from the group A rotavirus strains allowed identification of the four distinct NSP4 alleles; the Wa, KUN, AU-1, and EW alleles. On the other hand, the hydrophobicity profiles predicted by the Kyte–Doolittle algorithm for the group A rotavirus NSP4 amino acid sequences used in this study did not show the specific features according to each allele and the structural features of the group A rotavirus NSP4s were consistent irrespective of alleles; besides the enterotoxigenic activity of the NSP4s was also likely to be consistent irrespective of alleles. We speculate that the enterotoxigenic activity of the NSP4 is dependent on the consistent structural feature and the well-conserved putative receptor-binding site at residues 114–130.

MATERIALS AND METHODS

Virus strains

The rotavirus strains, EW, B-I, HI23, and R2, were grown in MA104 cells in the presence of 0.5 μg of trypsin (type IX, Sigma, St. Louis, MO) per milliliter. The virus particles were purified from infected MA104 cell cultures by pelleting at 36,000 rpm for 3 h in a Beckman type 45Ti rotor followed by sedimentation through 30% (w/v) su-

crose at 38,000 rpm for 3 h in a Beckman-type SW41Ti rotor.

Reverse transcription-polymerase chain reaction (RT-PCR), cloning, and sequencing

Genomic RNA was extracted with phenol-chloroform from purified virions and reverse transcribed with an NSP4 gene-specific 3′-terminal primer (EndG10: 5′-GGTCACATTAAGACCATTCC-3′) (Horie *et al.,* 1997). The cDNA was amplified by PCR with the same 3′-terminal primer and an NSP4 gene-specific 5′-terminal primer (1UG10: 5′-TTTTAAAAGTTCTGTTC-3′), cloned into pCR2.1 vector (Invitrogen, San Diego, CA), and sequenced on an ABI PRISM 377 automated DNA sequencer by the DyeDeoxy terminator method (Perkin Elmer, Norwalk, CT).

Three independent cDNA clones were sequenced at least twice on both strands, with an average sequence-run length for unambiguous reading from each primer of approximately 400 nt.

Sequence analysis and hydrophobicity profiling

The rotavirus NSP4 sequences used in this study are summarized in Table 1. For amino acid sequence comparisons and hydrophobicity profiles, we used only the amino acid sequences that were deduced from the open reading frame spanning nt 42–566, thus avoiding the effect of incorporated terminal primer sequences. The hydrophobicity profiles of the NSP4 amino acid sequences from 31 strains of group A rotaviruses were analyzed with a program in the GeneWorks Version 2.5 software package (IntelliGenetics, Campbell, CA) that utilized the Kyte and Doolittle algorithm, and the NSP4 sequences were aligned with the CLUSTAL W software package (Thompson *et al.,* 1994). Evolutionary distances, expressed as the number of amino acid substitutions per 100 sites (percentage divergence), between every pair of the NSP4 amino acid sequences were estimated with the ProtDist program in the Phylip 3.5c software package (Felsenstein, 1993). A phylogenetic tree based on the neighbor-joining method of Saitou and Nei (1987) was drawn with the N-J plot program in the CLUSTAL W software package (Thompson *et al.,* 1994).

Construction of recombinant expression vector

The truncated region corresponding to amino acid residues 86–175 of the EW NSP4 was generated by PCR with a site-specific 3′-primer (GCCGGATCCTCACAATGGCGCGATAACATC-3′; based on the sequence nt 563–546) and a site-specific 5′-primer (5′-GCCGGATCCAACGACTATCTCACAGATAAA-3′; based on the sequence nt 294–314), which contained a *Bam*HI recognition site. A GCC sequence was added at the 5′-terminus of both primers such that *Bam*HI could effectively cut the recognition site. The complementary sequence of the termination codon was introduced next to the 3′-terminus of the *Bam*HI recognition site in the site-specific 3′-primer. An amplified and *Bam*HI-cut cDNA fragment was cloned into a *Bam*HI-cut pGEX-4T-1 expression vector (Amersham Pharmacia Biotech, Buckinghamshire, U.K.).

Expression and purification of the glutathione S-transferase (GST) protein and the GST-amino acid residues 86–175 of EW NSP4 (GST-EW NSP4$_{86\text{-}175}$) fusion protein

The GST protein and the GST-EW NSP4$_{86\text{-}175}$ fusion protein were expressed and purified essentially according to Smith and Johnson (1988). Overnight cultures of *E. coli* strain DH5α, transformed with either the pGEX-4T-1 or with the recombinant pGEX-4T-1, were diluted 1:100 into fresh YT medium containing ampicillin (100 μg/ml) and grown to an OD_{600} of 0.3–0.4. Gene expression was induced by addition of isopropylthio-β-D-galactoside (Roche Diagnostics, Mannheim, Germany) to 0.1 mM and cells were incubated for a further 3 h. Bacteria were pelleted by centrifugation at 7000 rpm for 10 min at 4°C and resuspended in 1/20 vol of phosphate-buffered saline (PBS) with 0.5 mg lysozyme (Roche Diagnostics, Mannheim, Germany) per milliliter for 30 min on ice. After addition of dithiothreitol (Amersham Pharmacia Biotech, Buckinghamshire, U.K.) at a final concentration of 5 mM, cells were disrupted using a Bioruptor UCD-200TM (Cosmo Bio, Tokyo, Japan). Triton X-100 was added at a final concentration of 1% to the cell lysates. Debris was removed by centrifugation as described above. Typically, 40-ml aliquots of the clarified cell lysates were rocked overnight with 1 ml of a 50% glutathione–Sepharose 4B (Amersham Pharmacia Biotech, Buckinghamshire, U.K.) slurry in PBS, 1 mM NaN_3. Glutathione–Sepharose 4B was collected by centrifugation and washed three times with 40 vol of PBS. The GST protein and the GST-EW NSP4$_{86\text{-}175}$ fusion protein were eluted three times with an equal volume of 10 mM reduced glutathione (Sigma, St. Louis, MO) in 50 mM Tris–HCl adjusted to pH 8.0 (the glutathione elution buffer). These proteins were concentrated using a Centricon-10 (10 kDa cutoff) (Millipore Corp., Bedford, MA) and the protein concentrations were determined using a Protein assay reagent (Bio-Rad Laboratories, Hercules, CA) according to the method of Bradford. These proteins were sterile based on results of bacteriologic culturing in LB broth incubated at 37°C for 1 week and lacked endotoxins based on the experimental data using an Endotoxin kit (Sigma, St. Louis, MO).

SDS–PAGE analysis of GST-EW NSP4$_{86\text{-}175}$ fusion protein

Whole cell lysates containing the GST-EW NSP4$_{86\text{-}175}$ fusion protein, the supernatant (soluble) fraction, and the pellet (insoluble) fraction following sonication and cen-

trifugation of the whole cells containing the GST-EW NSP4$_{86-175}$ fusion protein were prepared. These were lysed in lysis buffer (1% TritonX-100 in PBS), mixed with Laemmli sample buffer, and separated by electrophoresis on a 10% SDS–polyacrylamide gel in a Mini-Protean II apparatus (Bio-Rad Laboratories, Hercules, CA) according to the manufacturer's instruction followed by staining with Coomassie brilliant blue R-250.

Western blot of GST-EW NSP4$_{86-175}$ fusion protein and GST protein

Aliquots of 0.05 nmol of the purified GST-EW NSP4$_{86-175}$ fusion protein and the purified GST protein were electrophoresed on a 10% SDS–polyacrylamide gel. Electrophoresed proteins were transferred to a polyvinylidene fluoride (PVDF) microporous membrane (Millipore Corp., Bedford, MA) using a TE22 Mighty Small Transphor apparatus (Amersham Pharmacia Biotech, Buckinghamshire, U.K.) according to the manufacturer's instruction. The PVDF microporous membrane was probed with an anti-GST antibody (Amersham Pharmacia Biotech, Buckinghamshire, U.K.). The GST protein–antibody complexes were reacted with horseradish peroxidase-conjugated anti-goat immunoglobulin and visualized using the chromogen containing 0.56 mM 4-chloro-1-naphthol with 0.0003% hydrogen peroxidase as an enzyme substrate.

Diarrhea induction in neonatal mice

Into groups of 5- to 6-day-old CD1 mice (Charles River Japan, Yokohama, Japan) were administered intraperitoneally 1 nmol of the GST-EW NSP4$_{86-175}$ fusion protein in 50 μl glutathione elution buffer, 1 nmol of the GST protein in 50 μl glutathione elution buffer, or 50 μl of the glutathione elution buffer. The neonatal mice were isolated and kept warm and humid. To determine the presence of diarrhea, we examined each neonatal mouse every 1 h for the first 8 h and at 12 and 24 h after inoculation by gently pressing the abdomen. The severity of diarrhea was classified into three categories; watery diarrhea, loose yellow stool, and ordinary stool. Loose yellow stool with some liquid was classified into the category "watery diarrhea." Loose yellow stool was not considered diarrhea. Statistical analysis was performed at the 5% level of significance using a Kruskal–Wallis rank test in Table 3.

ACKNOWLEDGMENTS

The authors acknowledge R. Ito and Y. Nakamura for their technical assistance in the preparation of viruses and RNA. This study was supported in part by a grant-in-aid for a general scientific study from the Ministry of Education, Science, Sport and Culture, Japan.

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